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Mitochondrial DNA sequence variation and phylogeography of oceanic insects (Hemiptera: Gerridae: *Halobates* spp.)

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Abstract Relatively few insects have invaded the marine environment, and only five species of sea skaters, *Halobates* Eschscholtz (Hemiptera: Gerridae), have successfully colonized the surface of the open ocean. All five species occur in the Pacific Ocean, *H. germanus* White also occurs in the Indian Ocean, whereas *H. micans* Eschscholtz is the only species found in the Atlantic Ocean. We sequenced a 780 bp long region of the mitochondrial cytochrome oxidase subunit I gene (COI) for a total of 66 specimens of the five oceanic *Halobates* species. Our purpose was to investigate the genetic variation within species and estimate the amount of gene flow between populations. We defined 27 haplotypes for *H. micans* and found that haplotype lineages from each of the major oceans occupied by this species are significantly different, having sequences containing five to seven unique base substitutions. We conclude that gene flow between populations of *H. micans* inhabiting the Atlantic, Pacific, and Indian Ocean is limited and hypothesize that these populations have been separated for 1 to 3 million years. Similarly, there may be limited gene flow between *H. germanus* populations found in the Pacific and Indian Ocean and between *H. sericeus* populations inhabiting the northern and southern parts of the Pacific Ocean. Finally, we discuss our findings in relation to recent hypotheses about the influence of oceanic diffusion on the distribution and population structure of oceanic *Halobates* spp.

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Introduction

Although unparalleled among metazoans in their evolutionary success on land and in freshwater, relatively few insects have invaded the intertidal zone of the marine environment (Cheng 1976). Only five species of sea skaters (Hemiptera, Gerridae), *Halobates germanus* White, *H. micans* Eschscholtz, *H. sericeus* Eschscholtz, *H. sobrinus* White, and *H. splendens* Witlaczil have successfully colonized the open ocean (Herring 1961; Andersen and Polhemus 1976; Andersen 1982, 1991; Cheng 1985), where adults and juveniles spend their entire life on the sea surface, always at some distance from land. They feed on other animals belonging to the pleustonic community and are themselves preyed upon by seabirds and pelagic fish (Cheng 1975; Cheng and Harrison 1983; Senta et al. 1993). Eggs are deposited on various floating objects (Lundbeck 1914; Andersen and Polhemus 1976; Cheng 1985), sometimes even on live heteropods (Seapy 1996).

The distributions of the five oceanic *Halobates* species are now well established (Cheng 1989). *H. micans* is widely distributed in the Atlantic, Indian, and Pacific Ocean. *H. sobrinus* and *H. splendens* are confined to the eastern Pacific Ocean. *H. germanus* occurs in the Red Sea, Indian Ocean, and western Pacific Ocean. Finally, *H. sericeus* is found throughout the Pacific Ocean except for a zone 15° north and south of the equator which is occupied by *H. micans*. Sea skaters only occur in tropical and subtropical waters where the winter temperature does not fall much below 20 °C. Their abundance on any given water mass was found to be correlated with sea-surface temperatures, with an optimum temperature range for the four eastern Pacific species (*H. micans*, *H. sericeus*, *H. sobrinus*, and *H. splendens*) of 24 to 28 °C (Cheng and Schulenberger 1980).

Herring (1961) considered the group of oceanic *Halobates* species to be monophyletic, but a reconstruction of the phylogeny of the genus based upon morphology (Andersen 1991) as well as mitochondrial

DNA sequences (Damgaard et al. 2000) shows that some oceanic species are more closely related to coastal *Halobates* species than they are to each other (Fig. 1 shows a simplified phylogenetic diagram for the genus with emphasis on the oceanic species). A group comprising by *H. micans*, *H. sobrinus*, and *H. splendens* is related to the coastal species *H. flaviventris* Eschscholtz (Indo-West Pacific) and *H. hawaiiensis* Usinger (Central Pacific); the species pair *H. germanus* and *H. sericeus* is related to *H. hayanus* White (Indo-West Pacific) and possibly a few other coastal species of this species group. Based upon the reconstructed phylogeny, Damgaard et al. (2000) concluded that the oceanic habit has evolved at least twice within the genus *Halobates*.

The purpose of the present study was to investigate genetic variation within the five oceanic *Halobates* species in order to estimate gene flow between populations inhabiting more than one ocean (*H. micans* and *H. germanus*) or different parts of the same ocean (*H. micans*, *H. sobrinus*, *H. splendens*, and *H. sericeus*). We discuss our findings in relation to potential barriers to gene flow, actual or in the past, as well as recent hypotheses about the influence of oceanic diffusion on the distribution and population structure of oceanic *Halobates* (Ikawa et al. 1998).

Materials and methods

Samples

Samples of oceanic *Halobates* for this study were collected from all three oceans, either by one of the authors (L. Cheng) or by

colleagues from various institutions during oceanic expeditions (for locality data, see Table 1). Specimens of Indian Ocean *H. germanus* and *H. micans* were collected specifically for our study by Dr. M. Baars of the Netherlands Institute for Sea Research (NIOZ) during two JGOFS cruises to the Arabian Sea in 1995. Sampling was carried out by using a neuston net. *Halobates* specimens were sorted on board ship, frozen live, and stored in liquid nitrogen until shipment to NIOZ. They were then identified to species and sex while still frozen, and DNA was extracted from individuals of representative samples. All other specimens were preserved in either 70% or 95% ethanol upon collection. We found no differences in the amounts of extractable DNA from *Halobates* specimens kept either at room temperature (20 °C), or stored in a freezer (−20 °C) (L. Cheng and A. Shedlock unpublished data).

DNA sequences and protocols

We amplified a region of the mitochondrial cytochrome oxidase subunit I gene (COI) for a total of 66 specimens of five oceanic *Halobates* species (Table 1). For most samples, we extracted mtDNA from alcohol-preserved adults, but for a few species, live specimens were killed in a −70 °C freezer and extracted on site. Except for *H. germanus*, we selected males and used only the thorax, which contains the powerful leg muscles. By removing the abdomen the risk of contamination with gut contents was also reduced. The head, abdomen, and limbs were stored in alcohol as voucher specimens. They are now deposited at the Zoological Museum, University of Copenhagen.

MtDNA was extracted by using the QiaAmp tissue kit protocol (QIAGEN Inc., Santa Clara, California) which included at least 2 h digestion of tissue with Proteinase K resulting in a volume of 300 µl DNA in solution. Polymerase chain reaction (PCR) amplifications were carried out in a thermal cycler in 51 µl of a cocktail containing 2 µl template, 5 µl of each primer (5 µM), 14 µl ddH₂O, 20 µl dNTPs (GATC 0.5 mM each), and 5 µl of Promega PCR-reaction buffer (15 mM MgCl₂). After a “hot start” with 2 min of denaturation at 94 °C, the reaction was paused at 72 °C and 0.2 µl *Taq* polymerase (5 U µl^{−1}) was added. Amplification parameters for each of the following 35 cycles were as follows: 94 °C for 1 min (denaturation), 45 °C for 1 min (annealing), and 72 °C for 1.5 min (extension).

The target sequence was 788 bp from the 3' half of the COI gene (corresponding to Site Nos. 2184 to 2971 in *Drosophila yakuba*) delimited by the primers Jerry (C1-J-2183) (5'CAA CAT TTA TTT TGA TTT TTT GG 3') (Simon et al. 1994), and a modified version of C1-N-2968 (Sperling et al. 1997) designated K866 (C1-N-2972) (5'GTA TTT CGT TAT AA/T/C/GG AA/GT GTT 3'). Because this sequence was too long to be sequenced in one step, two internal primers were designed for amplification with the endprimers, namely LimH2929 (C1-N-2609) (5'CGA ATA CTG CTC CTA TTG ATA 3') to work with Jerry, and Nils (C1-J-2387) (5'TCA CCA TCA ATA TTG TGA AC 3') to work with K866. These gave an overlap of 220 bp. The PCR-product was tested on a 2% NuSieve gel, stained with ethidium bromide, and sized with a ΦX174/HaeIII (Boehringer Mannheim) DNA ladder under UV-light. PCR products were cleaned of primers and unincorporated nucleotides with a QIAquick PCR Purification Kit (QIAGEN Inc.). Cycle sequencing was done using a Perkin Elmer/ABI Dye Terminator Cycle Sequencing Kit and run on a thermocycler using the profiles recommended by the kit manufacturers. The DNA sequence for each species was confirmed with both sense and anti-sense strands based on the same primers used in the PCR-amplification. Cycle sequencing products were cleaned using Centriscap columns, and sequenced using a Perkin Elmer ABI377 Automated Sequencer (Applied Biosystems Inc., Foster City, California). As the COI gene is protein coding with no insertions or deletions and relatively conserved amino acid sequences, alignment and contig construction was unproblematically performed in the program Sequencher (Gene Codes Corporation, Inc., Ann Arbor, Michigan). COI sequence data for specimens representing each of the five oceanic *Halobates* species are available from GenBank (accession numbers, see Damgaard et al. 2000).

Fig. 1 Phylogeny of *Halobates* spp. (simplified after Damgaard et al. 2000). Branches leading to oceanic species highlighted and species names shown in boldface. Number of species indicated for species groups. Hatched branch denotes clade composed of 20 coastal species

Table 1 *Halobates* spp. List of samples of oceanic species sequenced for COI mtDNA with haplotype assignment, locality, and sample data (LC Lanna Cheng collection; NIOZ Netherlands Institute for Sea Research)

Species (sample code)	Haplotype (no. of individuals)	Ocean	Location	Sample data	Year
<i>H. micans</i> (1a, 1b)	ma1 (1), ma3 (1)	W Atlantic	17N;66W	LC/EW28	1981
<i>H. micans</i> (91)	ma4 (1)	W Atlantic	17N;63W	LC/SEA1	1995
<i>H. micans</i> (92)	ma2 (1)	W Atlantic	16N;60W	LC/SEA2	1996
<i>H. micans</i> (136)	ma8 (1)	W Atlantic	14N;52W	LC/EW74	1981
<i>H. micans</i> (134)	ma7 (1)	C Atlantic	18N;40W	LC/EW63	1981
<i>H. micans</i> (132)	ma9 (1)	E Atlantic	23N;24W	LC/EW42	1981
<i>H. micans</i> (129)	ma5 (1)	E Atlantic	33N;17W	LC/EW23	1981
<i>H. micans</i> (130)	ma6 (1)	E Atlantic	30N;16W	LC/EW27	1981
<i>H. micans</i> (110)	mp5 (1)	W Pacific	23N;120E	LC/Taiwan	1995
<i>H. micans</i> (48, 49)	mp2 (1), mp8 (1)	W Pacific	13N;131E	LC/H95-144, 145	1995
<i>H. micans</i> (50, 51)	mp6 (1), mp7 (1)	W Pacific	13N;131E	LC/H95-159, 160	1995
<i>H. micans</i> (108)	mp4 (1)	W Pacific	14N;134E	LC/H94-23	1994
<i>H. micans</i> (107)	mp3 (1)	W Pacific	16N;137E	LC/H94-70	1994
<i>H. micans</i> (71)	mp8 (1)	E Pacific	1N;97W	LC/7701-3N	1979
<i>H. micans</i> (4)	mp2 (1)	E Pacific	4N;88W	LC/EW93	1981
<i>H. micans</i> (3)	mp1 (1)	E Pacific	6N;86W	LC/EW90	1981
<i>H. micans</i> (34)	mi4 (1)	W Indian	16N;58E	NIOZ/30S	1995
<i>H. micans</i> (33)	mi7 (1)	W Indian	15N;62E	NIOZ/28S	1995
<i>H. micans</i> (32, 31)	mi5 (1), mi6 (1)	W Indian	11N;52E	NIOZ/25S, 26S	1995
<i>H. micans</i> (15)	mi10 (1)	W Indian	10N;52E	NIOZ/7S	1995
<i>H. micans</i> (10, 90)	mi1 (1), mi9 (1)	W Indian	6N;55E	NIOZ/1H, H1	1995
<i>H. micans</i> (12, 11, 60)	mi2 (1), mi3 (1), mi8 (1)	W Indian	5N;49E	NIOZ/1S, 2S	1995
<i>H. splendens</i> (163)	sp2 (1)	E Pacific	1S;93W	LC/EW114	1982
<i>H. splendens</i> (5, 102)	sp1 (1), sp5 (1)	E Pacific	3S;96W	LC/EW118	1982
<i>H. splendens</i> (6)	sp5 (1)	E Pacific	5S;99W	LC/EW121	1982
<i>H. splendens</i> (164)	sp3 (1)	E Pacific	5S;100W	LC/EW122	1982
<i>H. splendens</i> (165)	sp4 (1)	E Pacific	6S;101W	LC/EW123	1982
<i>H. sobrinus</i> (70)	so1 (1)	E Pacific	14N;112W	LC/770113N	1979
<i>H. sobrinus</i> (69)	so2 (1)	E Pacific	6N;94W	LC/77019N	1979
<i>H. sobrinus</i> (95)	so1 (1)	E Pacific	5N;90W	LC/77017N	1979
<i>H. sobrinus</i> (66)	so3 (1)	E Pacific	4N;88W	LC/EW93	1982
<i>H. sobrinus</i> (65)	so1 (1)	E Pacific	6N;84W	LC/EW87	1982
<i>H. sobrinus</i> (2, 57, 64)	so1 (2), so2 (1)	E Pacific	6N;81W	LC/EW85, 86	1982
<i>H. germanus</i> (9)	ge2 (1)	S Pacific	18S;177W	LC/EW209	1982
<i>H. germanus</i> (115)	ge2 (1)	S Pacific	17S;179E	LC/Mana Is.	1981
<i>H. germanus</i> (47)	ge1 (1)	S Pacific	10S;175E	LC/H95-80	1995
<i>H. germanus</i> (25)	ge11 (1)	W Indian	23N;60E	NIOZ/17 T	1995
<i>H. germanus</i> (22)	ge7 (1)	W Indian	22N;60E	NIOZ/15S	1995
<i>H. germanus</i> (23, 24)	ge8 (1), ge10 (1)	W Indian	22N;61E	NIOZ/16S, 17S	1995
<i>H. germanus</i> (21)	ge6 (1)	W Indian	19N;58E	NIOZ/14S	1995
<i>H. germanus</i> (26, 27)	ge8 (2)	W Indian	17N;55E	NIOZ/20S, 21S	1995
<i>H. germanus</i> (19, 20)	ge4 (1), ge5 (1)	W Indian	16N;62E	NIOZ/12S, 13S	1995
<i>H. germanus</i> (28)	ge8 (1)	W Indian	16N;53E	NIOZ/22S	1995
<i>H. germanus</i> (29, 30)	ge8 (1), ge9 (1)	W Indian	14N;51E	NIOZ/23S, 24S	1995
<i>H. germanus</i> (17)	ge3 (1)	W Indian	13N;57E	NIOZ/9S	1995
<i>H. sericeus</i> (101)	se1 (1)	N Pacific	26N;159W	LC/Villareal	1995
<i>H. sericeus</i> (93)	se2 (1)	N Pacific	21N;158W	LC/Norpax 5	1979
<i>H. sericeus</i> (96)	se2 (1)	N Pacific	20N;158W	LC/Norpax 5	1979
<i>H. sericeus</i> (144)	se3 (1)	S Pacific	1S;106W	LC/EW126	1982
<i>H. sericeus</i> (94)	se3 (1)	S Pacific	7S;90W	LC/77015N	1979
<i>H. sericeus</i> (98)	se2 (1)	S Pacific	15S;150W	LC/Norpax 27	1979
<i>H. sericeus</i> (8)	se3 (1)	S Pacific	17S;118W	LC/EW139	1982
<i>H. sericeus</i> (150)	se3 (1)	S Pacific	24S;179E	LC/EW231	1982

Analytical procedure

Based upon COI nucleotide sequences, we defined a number of different haplotypes for each of the five oceanic *Halobates* species (Table 1), including *H. splendens* (sp1 to sp5), *H. sobrinus* (so1 to so3), *H. germanus* (ge1 to ge11) and *H. sericeus* (se1 to se3). For *H. micans*, 27 haplotypes were assigned symbols according to the ocean where the specimen came from, ma1 to ma9 (Atlantic Ocean), mp1 to mp8 (Pacific Ocean), and mi1 to mi10 (Indian Ocean). Various subsets of these haplotypes were subjected to neighbor-joining and maximum parsimony analyses using the program

PAUP* 4.0b2 (Swofford 1998) in combination with MacClade 3.05 (Maddison and Maddison 1992). Unweighted parsimony analyses were performed using heuristic search with 20 random-addition iterations. Two estimates of clade stability were calculated using PAUP*: bootstrap values (based on 500 replicates) and branch support (a.k.a. Bremer support or decay index; Bremer 1994).

Phylogeographical analyses were performed on data sets composed of all sequenced specimens of *Halobates micans*, *H. germanus*, and *H. sericeus*. For each species, we calculated uncorrected (*p*) nucleotide divergence (using PAUP*) for all specimen-pairs. We also measured the geographical distance between localities for

specimen-pairs from the Atlantic Ocean (*H. micans*), Pacific Ocean (*H. micans*, *H. sericeus*), and Indian Ocean (*H. germanus*, *H. micans*). To test the degree of correlation between nucleotide divergences and geographical distances, the Mantel test was used (Sokal and Rohlf 1995). The statistical significance of the observed test statistic was found by comparisons with 1000 random permutations of the geographical distance matrix using the program BIOMstat 3.2 (Rohlf and Slice 1996).

The geographic differentiation of *Halobates micans* haplotypes was also quantified using the analysis of molecular variation procedure (AMOVA; Excoffier et al. 1992) as implemented in the computer program ARLEQUIN 1.1 (Schneider et al. 1997). This procedure calculates standard variance components and an array of haplotypic correlation measures, referred to as Φ -statistics, for hierarchic subdivisions of a population. The limited number of regions sampled prevented a meaningful test of the hierarchical variance components. Instead, we examined the overall partitioning of molecular variance (using the matrix of uncorrected (p) divergences calculated with PAUP*) among the three oceans and between pair-wise comparisons of oceans. In this non-hierarchical model the statistic of primary interest is the Φ_{st} , defined as the correlation of random nucleotypes within an ocean relative to that of the entire species. The significance of the observed Φ_{st} values was tested using the matrix permutation procedure available in the program ARLEQUIN (with 1000 permutations).

Results

Nucleotide variation and divergence

We sequenced 29 specimens of *Halobates micans* sampled from the eastern, central, and western Atlantic Ocean, the eastern and western Pacific Ocean, and the eastern Indian Ocean (Table 1). A total of 85 nucleotide sites vary over a 780 bp region of COI mtDNA. Sequence variation is scattered across most of this region (Figs. 2, 3), except for an unvaried region of 62 bp toward the beginning of the sequence (Site Nos. 2226 to 2290). Most variation (91%) is restricted to the third-base position of triplet codons. No variation is observed in second-base positions, whereas a total of eight first-base positions (9%) show variation. Six of these are synonymous transitions as part of a leucine codon, whereas two are non-synonymous transitions between valine and isoleucine codons.

The intraspecific variation of *Halobates micans* (0 to 5.00%) is much larger than that observed among specimens of other species of *Halobates*, e.g. 0 to 0.77% in 13 specimens of *H. nereis* Herring and 0 to 1.54% in 16 specimens of *H. germanus*, and is more than half of the variation found when pairs of closely related species of *Halobates* are compared, e.g. *H. micans* and *H. splendens* (4.49 to 6.82%), *H. flaviventris* and *H. hawaiiensis* (6.41%), and *H. germanus* and *H. sericeus* (7.31 to 8.08%) (Damgaard et al. unpublished data).

Uncorrected (p) nucleotide divergences are higher for pair-wise comparisons among *Halobates micans* specimens from the Indian Ocean (Table 2; mean = 1.41%, SE = 0.37%, N = 45) than among specimens from the Atlantic Ocean (mean = 0.92%, SE = 0.27%, N = 36) or from the Pacific Ocean (mean = 0.81, SE = 0.32%, N = 45). Population genetic tests of differences among haplotype diversities and nucleotide diversities for pair-wise comparisons among specimens from the three oceans are, however, not statistically significant (Table 3).

We obtained much higher nucleotide divergences from pair-wise comparisons between *Halobates micans* specimens from different oceans. Specimens from the Atlantic and Pacific Ocean differ by 2.57 to 3.98% (mean = 3.44%, SE = 0.27%, N = 90), specimens from the Atlantic and Indian Ocean differ by 3.33 to 5.00% (mean = 4.16%, SE = 0.37%, N = 90), and specimens from the Pacific and Indian Ocean differ by 3.08 to 5.00% (mean = 4.19%, SE = 0.43%, N = 100). The AMOVA showed significant differentiation among oceans. The Φ_{st} statistic indicated that 73.62% of the molecular variance in the sequenced region of COI was accounted for by the three oceans. The permutation test for pair-wise comparisons between oceans, adjusted for multiple comparisons using the sequential Bonferroni method (Sokal and Rohlf 1995), showed that the nucleotide divergences among samples of *H. micans* from the Atlantic, Pacific, and Indian Ocean are all significant at $P < 0.05$.

We also examined the possible association between nucleotide divergence and geographical distance for

Fig. 2 *Halobates micans*. A 780 bp region from the 3' end of the COI mtDNA gene (Specimen Code 15, western Indian Ocean, Haplotype mi10). Variable sites underlined. Sites with non-synonymous substitutions marked with an asterisk. Nucleotide sites numbered as in *Drosophila yakuba* (Simon et al. 1994)

CCAGAAGTATATATTTTAATTTTACCAGGATTGGAAATTATCTCACATATTATTAGACAAGAAAGTGGTAAAAGAAA	[2260]
CGCATTTGGTTCAACAGGAATAATTTATGTCATATTAGCTATTGGTTTATTAGGATTATTGTATGAGCTCATCATATAT	[2340]
TTACAGTTGGAATGGACGTAGATACTCGAGCATATTTTACTTCAGCCACTATGATTATTGCTATTCCAACCTGGAATTAAA	[2420]
ATTTTTFAGTTGACTAGCAACTATTCATGGATCTATAATTAATTTATTCCTTCAATATTGAACATTAGGATTGTATT	[2500]
TTTATTTACAATTGGGGACTAACAGGTATTGTCTTAGCCAACTCATCAATTGATATTGTATTACATGACACTTACTATG	[2580]
TAGTAGCACATTTCATTATGTACTTTCAATAGGAGCAGTATTGGCCATTATAGCAGGATTATTCAGTGTATCCATTA	[2660]
TTTACAGGAATAACAATAAATCCAAATGACTAAAACTCAATTTTTCACATATGTTTATTGGAGTCAATTTGACCTTTT	[2740]
TCCACAACACTTTTATAGGATTAAGAGGTATACCACGACGATATTCAGATTATCCAGATATATATATCATGAAATGTAA	[2820]
TTTCATCAATTGGATCAACTATTTCATTTCTAGGAACATATATATTCATTATAATTATATGAGAAAGTATAGTATCCAAA	[2900]
CGAAAGGTTATATTTGTTATAAATATAAATTCAGAAATTGAATGATTACAAAATATCCACCA	[2963]

each composed of two haplotypes, are supported by bootstrap values above 50% in the consensus tree (Fig. 6; bootstrap values 57 and 94%, branch support 1 and 3). When translated to amino acids, one valine–isoleucine substitution is found in two haplotypes from the Pacific Ocean (shared with all nine haplotypes from the Atlantic Ocean).

The ten *Halobates micans* haplotypes from the Indian Ocean (mi1 to mi10) differ in 6 to 16 sites (0.8 to 2.1%). Four substitutions are shared with haplotypes from the Atlantic Ocean (Fig. 3) and seven substitutions with haplotypes from the Pacific Ocean (including one at a first-base position). Haplotypes from the Indian Ocean are better resolved in the consensus tree (Fig. 6) with four groups supported by bootstrap values above 50% (Fig. 6; bootstrap values 64, 66, 73, and 80%, branch support 1, 2, 2, and 3). When translated to amino acids, one isoleucine–valine substitution is found in five haplotypes (mi3 to mi5 and mi8 and mi9).

The small sample sizes for the eastern Pacific *Halobates splendens* (six specimens, five haplotypes) and *H. sobrinus* (eight specimens, three haplotypes) do not justify further analyses. The nucleotide variation (Fig. 4; Table 2) is apparently much lower in these two species than in *H. micans*.

We analyzed a data set composed of the 11 haplotypes of *Halobates germanus* and the three haplotypes of *H. sericeus* (Table 1) using the near-shore species *H. hayanus* as outgroup. Both a neighbor-joining tree and a strict consensus tree of 12 most parsimonious trees (Fig. 7; length = 114, CI = 0.878, RI = 0.976) keep together all *H. germanus* haplotypes (bootstrap value 100%, branch support 18). *H. germanus* haplotypes

from the southern part of the Pacific Ocean (Table 1, ge1 to ge2) and western Indian Ocean (ge3 to ge11) can be characterized by a total of four specific substitutions (Fig. 5). The strict consensus tree (Fig. 7) distinguishes the two haplotypes from the southern Pacific Ocean (bootstrap value 87%, branch support 2), whereas the group of nine haplotypes from the western Indian Ocean is unsupported. Both the neighbor-joining tree and the strict consensus tree keep together the three *H. sericeus* haplotypes (Fig. 7, bootstrap value 100%, branch support 27) and group two haplotypes (se1 and se2; bootstrap value 96%, branch support 2).

Discussion

In *Halobates micans*, the COI gene (or at least the 780 bp sequenced) is highly variable among haplotypes sampled in various parts of the distributional area of this pantropical species (see maps in Cheng 1989; Ikawa et al. 1998). In addition, haplotypes from each of the major oceans occupied by *H. micans* (the Atlantic, Indian, and Pacific Ocean) are significantly different, having sequences containing five to seven unique base substitutions. These include two non-synonymous substitutions which, translated to proteins, imply one amino acid difference only found in haplotypes from the Indian Ocean and one found in specimens from the Atlantic and Pacific Oceans. In terms of pair-wise similarity between COI sequences, our analyses show that haplotypes from the Atlantic Ocean are more similar to those from the Pacific Ocean than both are to haplotypes from the Indian Ocean. Assuming that such differences in COI sequences reflect present genetic isolation between populations and, indirectly, the time passed since the last separation of populations inhabiting major ocean basins, we hypothesize that populations of *H. micans* now inhabiting the Indian Ocean (or at least its western parts) became isolated from other populations of this species before the separation of populations inhabiting the Pacific and Atlantic Oceans. The observed genetic differences between these populations are, however, not correlated with morphological differences that justify a subspecific or specific differentiation of *H. micans* (Andersen et al. unpublished observations).

Since *Halobates splendens*, the sister species of *H. micans* (Fig. 1), is restricted to the southeastern part of the Pacific Ocean, *H. micans* probably originated somewhere in the Indo-Pacific and subsequently spread to the Atlantic Ocean (Andersen 1991, 1998). Several possible routes of dispersal have existed in the past (Andersen 1999). In the Early and Middle Eocene, the Mediterranean was connected with the Indo-Pacific Ocean by the Tethys Sea, located in present day Turkey, Iraq, and the Persian Gulf. In this period, the Tethys was a wide and continuous sea, probably with numerous islands and archipelagoes. Jaczewski (1972) hypothesized that the pantropical *H. micans* dispersed from the

Fig. 7 *Halobates* spp. Strict consensus tree of 12 most parsimonious trees for 11 haplotypes of *H. germanus* and 3 haplotypes of *H. sericeus* (with *H. hayanus* as outgroup). Bootstrap percentages (500 replicates) shown above branches and branch support (a.k.a. Bremer support or decay index) shown below branches. Search conditions as in Fig. 6. Geographical distribution of haplotypes indicated on right side of tree

Indo-Pacific to the Atlantic Ocean through this Tethys sea connection which was closed by the appearance of the Arabian Peninsula, 5 to 10 million years (Myr) ago (Smith et al. 1994). Although the finding of a fossil *Halobates* in 45-Myr-old, marine deposits in northern Italy (Andersen et al. 1994) indicates the presence of sea skaters in the Mediterranean as early as Middle Eocene, we hypothesize that the oceanic species evolved more recently than that.

As was also pointed out by Jaczewski (1972), there have been other, more recent routes of dispersal to the Atlantic Ocean, e.g. from the Indian Ocean around southern Africa, or from the Pacific Ocean through Mesoamerica, before the closure of the Isthmus of Panama, about 3 Myr ago (Knowlton and Weigt 1998). At present, dispersal of oceanic *Halobates* around the southern end of Africa is hampered by the presence of cold surface currents along the western coast of the African continent. On the other hand, surface temperatures in these parts of the Atlantic Ocean may have been higher in the past (Ricklefs and Latham 1993; Andersen 1999). However, the magnitude of nucleotide divergences between *H. micans* haplotypes from the Indian and Atlantic Oceans definitely speaks against a recent gene exchange between populations by dispersal around the southern end of Africa.

Brower (1994) suggested an almost linear relationship between mtDNA sequence divergences and age of splitting events for recently diverged arthropod taxa assuming an underlying constant substitution rate of 1.1 to 1.2% per Myr (which corresponds to about 2.3% sequence divergence per Myr). By applying these figures to our observed nucleotide divergences for samples of *Halobates micans*, the Atlantic populations may have been separated from the Pacific populations 1.1 to 1.8 Myr ago and from the Indian Ocean populations 1.4 to 2.2 Myr ago, or about the same time when the Pacific and Indian Ocean populations became separated. If the minimum age of separation between populations inhabiting the Atlantic and Pacific Oceans is 3 Myr (as dated by the emergence of the Isthmus of Panama; see Knowlton and Weigt 1998), the estimated substitution rate in *Halobates* is 0.4 to 0.7% per Myr or only about half the value estimated above.

The most probable explanation for this deviation is that *Halobates* diverge at a slower rate than the sample of arthropods used by Brower (1994). Sperling et al. (1997) found 2.8 to 3.3% COI nucleotide divergence between species-pairs of *Limnopus* water striders from the Palearctic and Nearctic regions. Assuming that these species were separated at least 1.8 Myr ago (before the onset of the Pleistocene), estimated substitution rates range from 0.8 to 0.9% per Myr. Similar rates are found in transberingian species-pairs of other freshwater Gerrids (Damgaard et al. unpublished observations).

Another, although less probable explanation is that the final closure of the Isthmus of Panama is of a more recent date than 3 Myr ago. Some authors have sug-

gested leakage or a final breach of the Isthmus as recent as 2 Myr ago (Cronin and Dowsett 1996). On the other hand, Knowlton and Weigt (1998) found that trans-isthmian species-pairs of snapping shrimps (*Alpheus* spp.) are often older than 3 Myr, which they attribute to variation in timing of the severance of gene flow across the Isthmus of Panama (e.g. due to ecological differences). The same may apply to other marine organisms. In particular, species confined to off-shore marine habitats like ocean skaters (*Halobates* spp.) are more likely than near-shore species to have diverged before the isthmian closure by an unknown span of time.

The relatively large substitution differences between *Halobates micans* haplotypes found in the Indian and Pacific Oceans (3.1 to 5.0%) suggest that the Indo-Malayan Archipelago constitutes a barrier for continuous gene flow between populations in these two oceans. This may also be the explanation for the genetic differences that separate haplotypes of *H. germanus* from the Indian and Pacific Oceans.

Based upon a theoretical investigation of the effects of oceanic diffusion on the distribution of oceanic *Halobates*, Ikawa et al. (1998) showed that an estimated radius of a "patch" of *Halobates* could be expanded by oceanic diffusion alone from an initial point of origin to 1250 km in 60 d. This distance is only 1/12 of the maximum distributional range of *H. micans* in the Pacific Ocean. Since eggs of oceanic *Halobates* may take > 1 month to hatch, nymphal development may take up to 70 d, and adults can live for at least 2 months (Cheng 1985), the descendants of a pair of individuals may be carried across the Pacific Ocean within the time required for several life cycles. Following these calculations, Ikawa et al. (1998) hypothesized that gene flow could occur by oceanic diffusion alone over the whole distributional range of a *Halobates* species. The results of the present investigation of nucleotide variation in oceanic *Halobates*, in particular those for *H. micans*, do not contradict this hypothesis. Thus, we found no significant association (i.e. positive correlation) between nucleotide divergence and geographical distance for the population of *H. germanus* inhabiting the western Indian Ocean or for *H. micans* populations inhabiting the Atlantic, Pacific, or Indian Ocean. In particular, we report two cases where *H. micans* individuals with the same haplotype are separated by >15 000 km across the Pacific Ocean.

Halobates sericeus has a disjunct amphitropical distribution in the Pacific Ocean. The northern and southern populations are separated along the equator by a relatively narrow zone where it is replaced by *H. micans* (see distribution map in Cheng 1989). Mixing of the two species is likely to occur only during seasonal weakenings of zonal equatorial currents (Cheng 1997). Although our results for *H. sericeus* are based upon a relatively small sample, the observed differences between haplotypes (including a non-synonymous substitution) lend support to the hypothesis that there is little or no genetic exchange between the northern and southern

populations (Cheng 1997). Further investigations using larger samples are, however, necessary to substantiate this.

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